The protective effect of palm tocotrienol-rich fraction against H_2O_2- induced oxidative stress in neonatal rat cardiomyocytes

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Background: Oxidative stress plays an important role in the pathogenesis of heart diseases. Antioxidants such as palm tocotrienol-rich fraction (TRF) can reduce oxidative stress, hence preventing and reducing the risk of heart disease. This study was aimed to determine the protective effects of TRF against hydrogen peroxide (H_2O_2) - induced oxidative stress in neonatal rat cardiomyocytes (NRCM). **Methods**: The NRCM were divided into five groups: (1) control, (2) cells treated with TRF (10 µg/ml) for 24 hours, (3) cells subjected to H_2O_2 (0.5 mM) for 30 minutes, (4) cells pre-treated with TRF, and (5) cells post-treated with TRF. The IC_{50} of H_2O_2 (0 – 5 mM) and the effective dose of TRF (0 – 25 µg/ml) were determined using the MTS cell viability assay. Meanwhile, ELISA was used to measure the level of reactive oxygen species (ROS). The presence of superoxides and H_2O_2 were detected by dihydroethidium and 5-(and-6) - carboxy -2′,7′-dichlorodihydrofluorescein diacetate respectively. Flowcytometry analysis was conducted to determine the presence of apoptosis and measure the mitochondrial membrane potential, whereby the former involved the use of Annexin V-FITC stain while the latter JC-1 stain. The gene expressions of antioxidant (*SOD*, *CAT*, *GPx*) and apoptosis (*Bax*, *Bcl-2*, *Caspase-3*) enzymes were studied using qRT -PCR. **Results**: The IC_{50} of H_2O_2 was 0.5 mM while the effective dose of TRF 10 µg/ml. The cells which were subjected to H_2O_2 showed a decrease in NRCM viability and significant increase ($p < 0.05$) in ROS production. LDH activity and green fluorescence intensity (which indicated mitochondrial depolarisation) were increased following H_2O_2 induction . With reference to the control, the H_2O_2- induced group had a higher percentage of late apoptotic cells, which was associated with the upregulation of the pro-apoptotic gene, *Bax*, and downregulation of the anti-apoptotic gene, *Bcl-2* ($p < 0.05$). H_2O_2 also upregulated *GPx* expression , apart from downregulating *CAT* and *Cu/Zn SOD* expression ($p < 0.05$). The pre- and post-treatment groups had increased cell viability and reduced ROS production. Pre-treatment with TRF protected the cell membranes and mitochondria from H_2O_2- induced injury, as reflected by the reduction in extracellular LDH activity and apoptosis (the latter of which was associated with the

downregulation of *Bax*). Meanwhile, the expression of *GPx*, *Cat*, and *Cu/Zn SOD* was reduced in the post-treatment group. **Conclusion**: By scavenging for ROS, palm TRF directly protects the cell membrane from H_2O_2- induced injury, leading to a decrease in oxidative stress. Thus, palm TRF maintains the mitochondrial membrane potential and prevents apoptosis secondary to decreased *Bax* expression.

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ABSTRACT

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Methods: The NRCM were divided into five groups: (1) control, (2) cells treated with TRF (10 µg/ml) for 24 hours, (3) cells subjected to H_2O_2 (0.5 mM) for 30 minutes, (4) cells pre-treated with TRF, and (5) cells post-treated with TRF. The IC_{50} of H_2O_2 (0 – 5 mM) and the effective dose of TRF (0 – 25 µg/ml) were determined using the MTS cell viability assay. Meanwhile, ELISA was used to measure the level of reactive oxygen species (ROS). The presence of superoxides and H_2O_2 were detected by dihydroethidium and 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate respectively. Flowcytometry analysis was conducted to determine the presence of apoptosis and measure the mitochondrial membrane potential, whereby the former involved the use of Annexin V-FITC stain while the latter JC-1 stain. The gene expressions of antioxidant (*SOD*, *CAT*, *GPx*) and apoptosis (*Bax*, *Bcl-2*, *Caspase-3*) enzymes were studied using qRT-PCR.

Results: The IC_{50} of H_2O_2 was 0.5 mM while the effective dose of TRF 10 µg/ml. The cells which were subjected to H_2O_2 showed a decrease in NRCM viability and significant increase ($p < 0.05$) in ROS production. LDH activity and green fluorescence intensity (which indicated mitochondrial depolarisation) were increased following H_2O_2 induction. With reference to the control, the H_2O_2-induced group had a higher percentage of late apoptotic cells, which was associated with the upregulation of the pro-apoptotic gene, *Bax*, and downregulation of the anti-apoptotic gene, *Bcl-2*

($p < 0.05$). H_2O_2 also upregulated *GPx* expression, apart from downregulating *CAT* and *Cu/Zn SOD* expression ($p < 0.05$). The pre- and post-treatment groups had increased cell viability and reduced ROS production. Pre-treatment with TRF protected the cell membranes and mitochondria from H_2O_2-induced injury, as reflected by the reduction in extracellular LDH activity and apoptosis (the latter of which was associated with the downregulation of *Bax*). Meanwhile, the expression of *GPx*, *Cat*, and *Cu/Zn SOD* was reduced in the post-treatment group.

Conclusion: By scavenging for ROS, palm TRF directly protects the cell membrane from H_2O_2-induced injury, leading to a decrease in oxidative stress. Thus, palm TRF maintains the mitochondrial membrane potential and prevents apoptosis secondary to decreased *Bax* expression.

Keywords: TRF, Cardiomyocytes, Oxidative stress, H_2O_2, Oxidative damage

INTRODUCTION

Cardiovascular disease is one of the most prevalent ailments associated with high morbidity and mortality in both developing as well as developed countries (WHO, 2016). Studies have reported that oxidative stress plays a central role in the pathophysiology of heart diseases and causes cell death (Taverne et al., 2013). The accumulation of reactive oxygen species (ROS) may increase oxidative stress and cause detrimental modifications in cellular macromolecules. Examples of such modifications include lipid peroxidation, DNA damage, mitochondrial dysfunction, and enzymatic activity loss, all of which can lead to necrosis and/ or apoptosis (Biswas, 2016).

Stimuli such as oxidative stress and hypoxia give rise to changes in the mitochondrial membrane permeability, hence initiating the apoptotic mitochondrial pathway. The release of pro-

apoptotic proteins from the mitochondria into the cytosol is regulated by the Bcl-2 protein family, whose function is to control the permeability of mitochondrial membranes (Webster, 2012). An enzyme of the terminal apoptotic pathway is caspase-3, whereby its level of expression may indicate the size of the heart infarct (Condorelli et al., 2001).

ROS such as superoxide anions (O^{2-}), hydroxyl radicals (OH^{-}), and hydrogen peroxide ions (H_2O_2) are produced as part of physiological processes. The levels of ROS are controlled by antioxidant enzymes like catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD). They catalyse the conversion of these ROS to less-toxic products, apart from protecting cells against free radical-induced damage (Lobo et al., 2010).

The prevention of cardiomyocytes from damage and death is very important in light of the fact that post-mitotic (adult) cardiomyocytes have a reduced ability to undergo mitosis. As such, to overcome the workload of the heart, the existing cells have to become hypertrophic (Woodcock & Matkovich, 2005). In cardiac disease, the loss of cardiomyocytes weakens the contractile power of the heart (Tham et al., 2015). Therefore, interventions which involve antioxidants or natural compounds that have free radical-scavenging activities may provide beneficial effects against oxidative stress.

Studies in humans and animal models have revealed that vitamin E possesses antioxidant, anticancer, anti-inflammatory, antimicrobial activities, and other biological activities, apart from protecting the cardiovascular system (Galli & Azzi, 2010; Vasanthi et al., 2012; Wali et al., 2009). Vitamin E has been suggested to be a valuable compound with many medical applications It is a fat-soluble vitamin, which is composed of naturally-occurring α-, β-, γ-, and δ-tocopherols as well as -tocotrienols (Fu et al., 2014). Tocotrienol-rich fraction (TRF) refers to the fraction of palm oil

that consists mainly of a mixture of a α-, β-, γ-, and δ-tocotrienols as well as some α–tocopherols (Srivastava & Gupta 2006).

In this study, the effects of palm TRF on the H_2O_2-induced oxidative status and apoptosis of neonatal rat cardiomyocytes (NRCM) were determined.

MATERIALS AND METHODS

Isolation of neonatal rat cardiomyocytes (NRCM)

Using a modified protocol which was described by Salameh and Dhein (2005), NRCM were isolated from 1- to 2-day old Sprague-Dawley rats. The experimental protocol was approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (FP/BIOK/2012/ZAKIAH/18-JULY/450-APRIL-2013-APRIL-2016-AR-CAT2). With a pair of scissors, neonatal rat ventricles were cut into small pieces of about 1 mm length and stored in a cold ADS buffer. Then, all the tissues were enzymatically digested by collagenase type II (Worthington) and pancreatin (Sigma) for 4 to 5 times in shaker incubator at 37°C. After each cycle, the supernatant (which contained the isolated cells) was collected and suspended with fetal bovine serum (FBS). All supernatant from the cycles were then pooled and centrifuged at 800 rpm for 5 minutes, after which they were removed as well as resuspended in media containing DMEM, M199, 10% horse serum, 5% FBS, 100 U/L of streptomycin, and 100 U/L of penicillin. Pre-plating was performed by incubating the cells for 45 minutes in a cell culture flask at 37°C in a humidified atmosphere containing 5% CO_2. This was done in order to reduce contamination by fibroblasts and to obtain cardiomyocytes of high purity. Subsequently, the supernatant was collected and centrifuged at 800 rpm for 5 min. The resultant cell pellets were resuspended overnight in the mentioned media before being

transferred into media containing 5% FBS. The NRCM were seeded in experimental conditions at a density of 2 x 10^4 cells/well in the 96-well plate and 4 x 10^5 cells/well in the 6-well plate. They were then cultured for 3 to 4 days until synchronized beating NRCM were obtained.

Experimental group

The experiment proceeded with the treatment of the cells according to their groups: control group: NRCM were incubated in media; H_2O_2 group: NRCM were subjected to 0.5 mM H_2O_2 for 30 minutes; palm TRF group: NRCM were supplemented with 10 µg/ml palm TRF for 24 hours; pre-treatment group: NRCM were supplemented with palm TRF (10 µg/ml) for 24 hours before they were subjected to 0.5 mM H_2O_2 for 30 minutes; and post-treatment group: NRCM were supplemented with palm TRF after being subjected to 0.5 mM H_2O_2.

MTS assay

Various concentrations of H_2O_2 and palm TRF were used to treat the cells for 30 minutes and 24 hours respectively. The degree of cytotoxicity was measured via CellTiter 96® Aqueous Nonradioactive Cell Proliferation Assay (MTS; Promega, USA) according to the manufacturer's protocol. Briefly, 20 µl of MTS solution was mixed with 100 µl of media before being added to each well and incubated for 2 hours. Using a microtiter plate reader, the absorbance of formazan MTS was measured at a wavelength of 490 nm (VersaMax Molecular Devices, USA). The optimum dose of the treatment was used for subsequent experiments.

Reactive Oxygen Species (ROS) Generation

5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) and dihydroethidium (DHE) (Molecular Probes, USA) were used to assess the generation of oxidants

in NRCM. Carboxy-H_2DCFDA was oxidised by H_2O_2, giving rise to hydroxyl radicals or peroxynitrite. Meanwhile, DHE detected the production of superoxides. In terms of the procedure, NRCM were incubated in 40 µM of carboxy-H_2DCFDA and 20 µM of DHE for 45 minutes. After that, the cells were washed with PBS and the intensity of fluorescence measured using a microplate reader (Infinite® 200, Tecan, USA) at an excitation/emission wavelength (Ex/Em) of 488 – 521 nm and 518 – 600 nm respectively.

LDH

The cells were cultured in 6-well plates at a density of 4 x 10^5 cells/well. After treatment, the supernatant was collected and measured for their LDH activities via a detection kit which was utilized according to the manufacturer's instruction (Sigma, USA). LDH activity was expressed as international units per liter (IU/L).

Mitochondrial Membrane Potential ($\Delta\Psi_m$)

JC-1 staining was employed to assess $\Delta\Psi_m$, which was a marker of mitochondrial oxidative phosphorylation activity as previously described (Nowak et al., 2012). JC-1 is a lipophilic and cationic dye that permeates the plasma as well as mitochondrial membranes of cells. A low JC-1 ratio indicates the presence of a low amount of the aggregated form of JC-1 in the mitochondria, which correlates with a high level of ROS. Fluorescence was determined by flow cytometry (FACSVerse; BD Biosciences, San Jose, CA), 488-nm argon-ion laser. JC-1 monomers (green) and J-aggregates (red) were detected in FL1 (emission, 525 nm) and FL2 (emission, 590 nm) channels respectively. $\Delta\Psi_m$ was presented as the ratio of the fluorescence intensity of J-aggregates to that of J-monomers. For observation, the same staining protocol was applied. The cells were

then seen under a fluorescence microscope (EVOS FL digital inverted microscope, Thermo Fisher Scientific, USA).

Apoptosis

Annexin V-FITC Apoptosis Detection Kit (BD Pharmigen, USA) was used for apoptosis profiling. The cells were washed with PBS three times and suspended in 100 µl of binding buffer. Staining was done with 5 µl of FITC-conjugated Annexin V and 10 µl of PI, after which 400 µl of binding buffer was added as per the manufacturer's instructions. The percentages of both dyes were analysed by flow cytometry (FACSVerse, Becton-Dickinson, USA). Annexin V-FITC-positive and PI-negative cells indicated early apoptosis, while double-stained ones late apoptosis.

Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA extraction was performed using TRIzol (Invitrogen). 2 mg of total RNA was reverse-transcribed using the SuperScript First-Strand Synthesis System (Invitrogen). cDNA was synthesised from isolated RNA, and the cycle time (Ct) values were determined by real-time RT-PCR which utilised the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), the iQ5 Real-Time PCR Detection System, and an analytic software (Bio-Rad, USA) as previously described (Sun et al., 2010). The primers were designed using the Applied Biosystems Primer Express Software (version 2.0), and the primer sequences shown in Table I. The relative expression value was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistical analyses were performed using the SPSS 16.0 software (IBM, USA). The data was expressed as means ± standard deviations (mean ± SD) of three replicates. The results for all the tests were considered to be statistically significant if $p < 0.05$. ANOVA was used to analyse multiple groups, after which a post-hoc test was performed.

RESULTS

Effects of H_2O_2 and palm TRF on cell viability

Exposure to H_2O_2 concentrations of 0.5 mM and above significantly reduced the viability of the cells relative to the control (Fig 1A). In other words, the IC_{50} of H_2O_2 for NRCM was 0.5 mM. Pre-treatment with palm TRF of concentrations 10, 15, and 25 µg/ml significantly increased the cell viability from 54 ± 2.0% to 72 ± 5.3%, 71 ± 2.3%, and 70 ± 3.5% respectively (Fig. 1B). However, there was no significant difference when the cells were treated with 40 µg/ml of palm TRF. Meanwhile, when the cells were post-treated with the same concentrations as those of pre-treatment, their viability significantly increased to 96 ± 4%, 91 ± 3.5% and 93 ± 7.02% respectively for with palm TRF. Post-treatment with 40 µg/ml of palm TRF also significantly increased the cells' viability to 82 ± 9.5%. These results showed that both pre- and post-treatment with palm TRF had the ability to protect NRCM from oxidative stress. Owing to the fact that higher concentrations of palm TRF might be cytotoxic, a concentration of 10 µg/ml was chosen for the following experiment.

Effects of palm TRF on ROS production

The intensities of the carboxy-H_2DCFDA and DHE-stains were increased in the H_2O_2-treated cells as compared to control group ($p < 0.05$) (Fig. 2). On the other hand, pre- and post-treatment with

palm TRF significantly reduced the intensities of both stains, hence indicating a reduction in the amount of intracellular ROS production. The cells which were treated with palm TRF alone had a decreased intensity of DHE stain vis-à-vis the control.

Effects of palm TRF on LDH activity

LDH activity is widely used as a marker of cellular injury and necrosis. In this study, H_2O_2 increased the LDH activity of NRCM to 0.098 ± 0.01 U/ml ($p < 0.05$) as compared to the control (Fig. 3). Pre-treatment with palm TRF for 24 hours reduced LDH activity to 0.068 ± 0.001 U/ml. However, cells which were post-treated with palm TRF demonstrated an increase in LDH activity (0.136 ± 0.009 U/ml) relative to those which were treated by H_2O_2 only.

Effects of palm TRF on mitochondrial membrane potential changes

JC-1 staining of NRCM gave rise to a characteristic pattern of hypopolarized (green fluorescence of monomers) and hyperpolarized (red fluorescence of J-aggregates) mitochondria (Fig. 4A). In the control and palm TRF-treated groups, the intensity of the red fluorescence (J-aggregates) was higher than that of the green (J-monomers). Exposure to H_2O_2 increased the intensity of the green fluorescence, hence indicating mitochondrial depolarisation. On the contrary, the red fluorescence intensity was increased in the pre-treated group as compared to H_2O_2 group, thus demonstrating the protective effect of palm TRF. The cells which were post-treated with palm TRF showed a higher intensity of green fluorescence, which indicated mitochondrial membrane injury. Figure 4B shows the ratios of JC-1 aggregates to JC-1 monomers in NRCM. From there, it can be seen that the ratio was lower in the H_2O_2 group vis-à-vis the control. Palm TRF had the ability to increase the ratio relative to that of the control group. Pre-treatment with palm TRF increased the

mitochondrial membrane potential (MMP), but the aforementioned ratio was lower in the post-treated cells than the H_2O_2 group.

Effects of palm TRF on apoptosis

H_2O_2 increased the percentage of late apoptotic cells as compared to the control group ($p < 0.05$) (Fig. 5), while pre-treatment with palm TRF reduced the said percentage ($p < 0.05$). However, post-treated cells had an increased percentage of both early and late apoptotic cells relative to the H_2O_2 group ($p < 0.05$).

Effects of palm TRF on antioxidant enzyme gene expressions

With respect to the control, H_2O_2 and palm TRF upregulated *GPx1* (Fig. 6A) expression but downregulated that of *CAT* (Fig. 6B) and *Cu/Zn SOD* (Fig. 6C) ($p<0.05$). Pre-treatment with palm TRF showed no significant changes in the gene expressions. However, the mRNA expression of all the antioxidant enzymes (Fig. 6A-C) was downregulated in the post-treatment group as compared to H_2O_2 group ($p < 0.05$). *Mn-SOD* (Fig. 6D) expression was not affected by treatment.

Effects of palm TRF on apoptosis gene expression

Relative to the control, H_2O_2 upregulated the pro-apoptotic gene *Bax* and downregulated the anti-apoptotic gene *Bcl-2* ($p<0.05$) (Fig. 7A and 7C). Meanwhile, palm TRF downregulated *Bax, Caspase-3*, and *Bcl-2*. Pre-treatment with palm TRF decreased the mRNA expression of *Bax* and *Bcl-2* ($p < 0.05$) but not C*aspase-3* expression. However, the expression of *Bax* was increased while *Caspase-3* and *Bcl-2* decreased in the post-treatment group as compared to the H_2O_2 group, ($p < 0.05$).

DISCUSSION

Cardiomyocytes are prone for oxidative stress as ROS are actively produced as a side product of mitochondrial oxidative phosphorylation. Energy produced from oxidative phosphorylation is very important for heartbeat and contraction (Andersson et al., 2011). ROS give rise to oxidative stress and are a major contributor to cell death. Oxidative stress has been widely implicated in cellular damage and progression of cardiovascular diseases such as atherosclerosis, hypertension, heart failure, and myocardial infarction (Dikalova et al., 2010; Sugamura & Keaney, 2011). Low antioxidant availability in cardiomyocytes subjects them to oxidative damage. As such, vitamin E has been widely studied for their ability to reverse the effects of ROS, thereby protecting the cells from oxidative damage and death (Wu et al., 2010).

H_2O_2 that induces oxidative damage (Akyol et al., 2014) led to a reduction in the viability of NRCM in this study. High levels of ROS production, as indicated by increased staining by carboxy-H_2DCFDA and DHE, may lead to lipid peroxidation as well as cell membrane damage. Fenton reaction can also contribute to increased ROS production following the conversion of H_2O_2 to hydroxyl radicals (Bayeva et al., 2013). This suggests that H_2O_2 traverses the cell membrane and initiates a cascade of biochemical reactions which result in the accumulation of intracellular free radicals (Shao et al., 2004). Previous studies have proposed that the increase in lipid peroxidation is directly proportional to that of LDH activity (Hrelia et al., 2002) in the extracellular fluid, hence indicating myocardial cell membrane damage. Elevation of LDH activity usually denotes irreversible cardiomyocyte injury (Kourouma et al., 2015).

ROS, which include superoxide, hydroperoxyl, and hydroxyl radicals, are very reactive and unstable. While H_2O_2 is non-radical, it is still classified as a ROS because of its high oxidative

283 reactivity (Dröge, 2002). ROS are generated both intracellularly and extracellularly. Intracellular
284 ROS are predominantly produced during the activation of the mitochondrial respiratory chain
285 (Brand et al., 2004). In the process of ATP production, electrons leak from the mitochondrial
286 electron transport chain and formed anionic superoxide radicals (Andreyev et al., 2005). Some
287 consequences of ROS accumulation include ischemia and reperfusion injury, which in turn lead to
288 mitochondrial dysfunction in heart cells (Granger & Kvietys, 2015; Perrelli et al., 2011).
289 Superoxide radicals can react with each other spontaneously or form H_2O_2 in a reaction catalysed
290 by superoxide dismutase. ROS attack cell biomolecules such as DNA, lipid, and protein, thus
291 giving rise to oxidative damage (Birben et al., 2012). ROS-induced damage to the mitochondrial
292 membrane lipid disrupts the membrane integrity and permeability, apart from causing depolarising
293 alterations in the membrane potential (Lane et al., 2015). These in turn lead to cell membrane
294 injury and damage, culminating in the leakage of the cellular contents into the cytoplasm (Webster,
295 2012).

296 Furthermore, the products of lipid peroxidation act as uncouplers of respiratory chain
297 phosphorylation within the mitochondria in light of an increase in the permeability of the internal
298 mitochondrial membrane for protons. This mechanism creates a proton concentration equilibrium
299 at both sides of the internal mitochondrial membrane (Nagano et al., 2012). Another study also
300 reported that the inner mitochondrial membrane consists of unsaturated cardiolipin that is highly
301 vulnerable to peroxidation which results in altered functions in the aged heart (Lesnefsky &
302 Hoppel, 2008). Reactive aldehydes such as malonaldehydes (MDA) and 4-hydroxyhexenal (4-
303 HNE) are highly-reactive lipid peroxidation products (LPPs) which are suggested to play a role in
304 the pathogenesis of cardiovascular disease (Riahi et al., 2010). These LPPs attack the protein
305 channels in the cell membrane, leading to the accumulation of calcium ions in cardiomyocytes.

306 Subsequently, the mitochondrial permeability transition pores open, and this increases the risk of
307 heart failure (Negre-Salvayre et al., 2010; Uchida, 2000).

308 Mitochondrial dysfunction is an important factor in the pathogenesis of heart failure (Ide
309 et al., 2001; Rosca & Hoppel, 2013). Changes in mitochondrial function lead to increased ROS
310 levels and cellular homeostasis disruption, which in turn result in cardiomyocyte dysfunction and
311 eventually, activation of cellular destruction pathways (Tsutsui et al., 2008). This study has shown
312 that H_2O_2 gave rise to depolarisation of the mitochondrial membrane potential ($\Delta\psi m$) and
313 apoptosis, as reflected by the upregulation of *Bax* (a pro-apoptotic gene) and downregulation of
314 *Bcl-2* (an anti-apoptotic gene). However, the process of apoptosis may have occurred through a
315 pathway other than the caspase pathway, or that necrosis could have taken place instead of
316 apoptosis owing to the fact that the percentage of late apoptotic cells was increased with no
317 significant changes in the caspase-3 mRNA expression. Previous studies have shown that the
318 depolarisation of mitochondrial membranes caused depletion of energy due to decreased levels of
319 ATP generation, which could eventually change the mode of cell death from apoptosis to necrosis
320 (Nakamura et al., 2010; Tatsumi et al., 2003). Cardiomyocytes are reported to undergo apoptosis
321 in patients suffering from myocardial infarction, diabetic cardiomyopathy, and end-stage
322 congestive heart failure (Kuethe et al., 2007; Narula et al., 1999). Also, alterations in mitochondrial
323 function have been observed in studies on heart failure in humans (Sebastiani et al., 2007) and
324 animal models (Goh et al., 2015). These effects seem to be caused by changes in the expression of
325 proteins, which might be related to the decreased capacity to oxidise fatty acid substrates often
326 seen in heart failure (Lemieux et al., 2011)

327 When ROS levels are high, *GPx1* will be upregulated to detoxify H_2O_2 and protect cells
328 from oxidative mitochondrial damage. Studied by Thu et al. (2010) showed that the mitochondrial

membrane potential is lost following a decrease in the expression of the oxidative phosphorylation protein in *GPx1*(-/-) of mice hearts. *GPx1* is produced in all tissues and expressed in both cytosolic as well as mitochondrial matrix. The lack of *GPx1* makes an individual at risk of atherosclerosis and cardiovascular disease (Shiomi et al., 2004)

Tocotrienols are found more abundantly in palm TRF than in the oils of other plants. Palm TRF consists of 30% tocopherols and 70% tocotrienols (Sambanthamurthi et al., 2000). Tocotrienols have greater antioxidant activity than tocopherols (Ali & Woodman, 2015); studies have also suggested that the former have a cardioprotective effect in light of their ability to protect mitochondria from oxidative stress (Kamat & Devasagayam, 1995; Nowak et al., 2012). In this research, TRF given before (pre-treatment) and after (post-treatment) H_2O_2 induction successfully restored the viability of NRCM by reducing ROS generation. Yam et al. (2009) have also demonstrated the protective effect of TRF on macrophages. Other studies have reported that the supplementation of TRF increased the viability of senescent myoblasts (Khor et al., 2017).

TRF scavenges ROS by donating electrons to the free radicals, hence inhibiting the chain initiation and breaking the chain propagation (Lamichhane et al., 2013; Sharma et al., 2012). These prevent membrane lipid peroxidation that results in injury to membranes and leakage of functional enzymes (such as LDH) or cell contents into the cytoplasm. This finding is in line with that of Sharikabad et al. (2004), who reported a reduction in LDH leakage in light of declining ROS levels. The same effect has been demonstrated in H_2O_2-induced neuron cells, whereby lipid peroxidation was inhibited by TRF treatment (Fukui et al., 2012). This could be due to the action of TRF in maintaining the membrane integrity, thereby restricting the leakage of this enzyme. There are reports on the prevention of erythrocyte lysis by vitamin E supplementation; the lipid-solubility of the vitamin enables it to easily diffuse into the lipid membrane and stabilising it (Howard et al.,

2011). These protective effects were observed in the cells treated with TRF prior to administration oxidative stress.

Pre-treatment with TRF seemed to directly protect cardiomyocytes through intracellular ROS scavenging because no changes were observed in the *GPx1* expression, contrary to the finding in the H_2O_2-induced cells whereby the gene was upregulated. The depolarisation of $\Delta\psi m$ by H_2O_2 was also prevented by pre-treatment with TRF. Previous studies have shown that γ-tocotrienol protects mitochondria from oxidative stress (Nowak et al., 2012), which in turn reduces the occurrence of cell death, especially necrosis (Miura et al., 2010). In this study, TRF was shown to lower the percentage of late apoptotic cells associates with the reduction in *Bax* expression.

However, detrimental changes in NRCM secondary to higher levels of ROS cannot be prevented, as reflected by the findings in the post-treatment group (Jilanchi et al., 2013). As with this study, the oxidative damage occurring after H_2O_2 induction most probably could not be repaired by TRF, hence giving rise to progressive cell death (Han et al., 2004). This was supported by the presence of an extremely high level of late apoptotic cells in the post-treatment group, apart from increased expression of *Bax* and reduced expression of *Bcl-2* as well as *caspase-3*. A decrease in *caspase-3* expression does not affect the percentage of cells undergoing death. This may be due to the time-dependent cell death after H_2O_2 withdrawal. The study by Han et al. (2004) reported that intracellular ROS levels further increased even after H_2O_2 withdrawal, which in turn led to mitochondrial membrane depolarisation and cell death.

Even though, that the post-treatment intracellular ROS level was low, this could have been due to direct scavenging of ROS by TRF instead of increased *GPx* or other antioxidant gene expression. Interestingly, in the cells treated with TRF but not H_2O_2, *GPx* was still upregulated, thus indicating that TRF preferentially targets this gene in the cardiomyocytes to enable effective

removal of H_2O_2 from the system. This action could have been adequate to counteract the existing ROS as there was downregulation of *CAT* and *Cu/Zn SOD* in the TRF group.

CONCLUSION

By scavenging for ROS, palm TRF protects NRCM from oxidative damage rather than treating the same. It restores the mitochondrial membrane potential, thus decreasing cell death by attenuating the expression of *Bax*.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interest

The authors declare that they have no competing interests.

Author Contributions

- Noor Shareena Aisha Abdul Khalid conceived, designed and performed the experiments, apart from analysing the data, writing the paper, as well as preparing figures and/ or tables.

• Zakiah Jubri analysed the data and reviewed the drafts of the paper.

Animal Ethics

Ethical clearance for this study was obtained from the Universiti Kebangsaan Malaysia Animal Ethics Committee (reference number: FP/BIOK/2012/ZAKIAH/18-JULY/450-APRIL-2013-APRIL-2016-AR-CAT2)

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Figure 1(on next page)

Cell viability in NRCM

A) Effect of different concentration of H_2O_2 (0.5-5 mM) on the cell viability. **B)** Effect of TRF and H_2O_2 IC_{50} on the cell viability. Data are expressed as mean ± SD from three independent experiments (N=3). * indicates significant difference $p < 0.05$ compared to control . [a] indicates significant difference $p<0.05$ compared to control group and [b] indicates significant difference $p<0.05$ compared to H_2O_2 group .

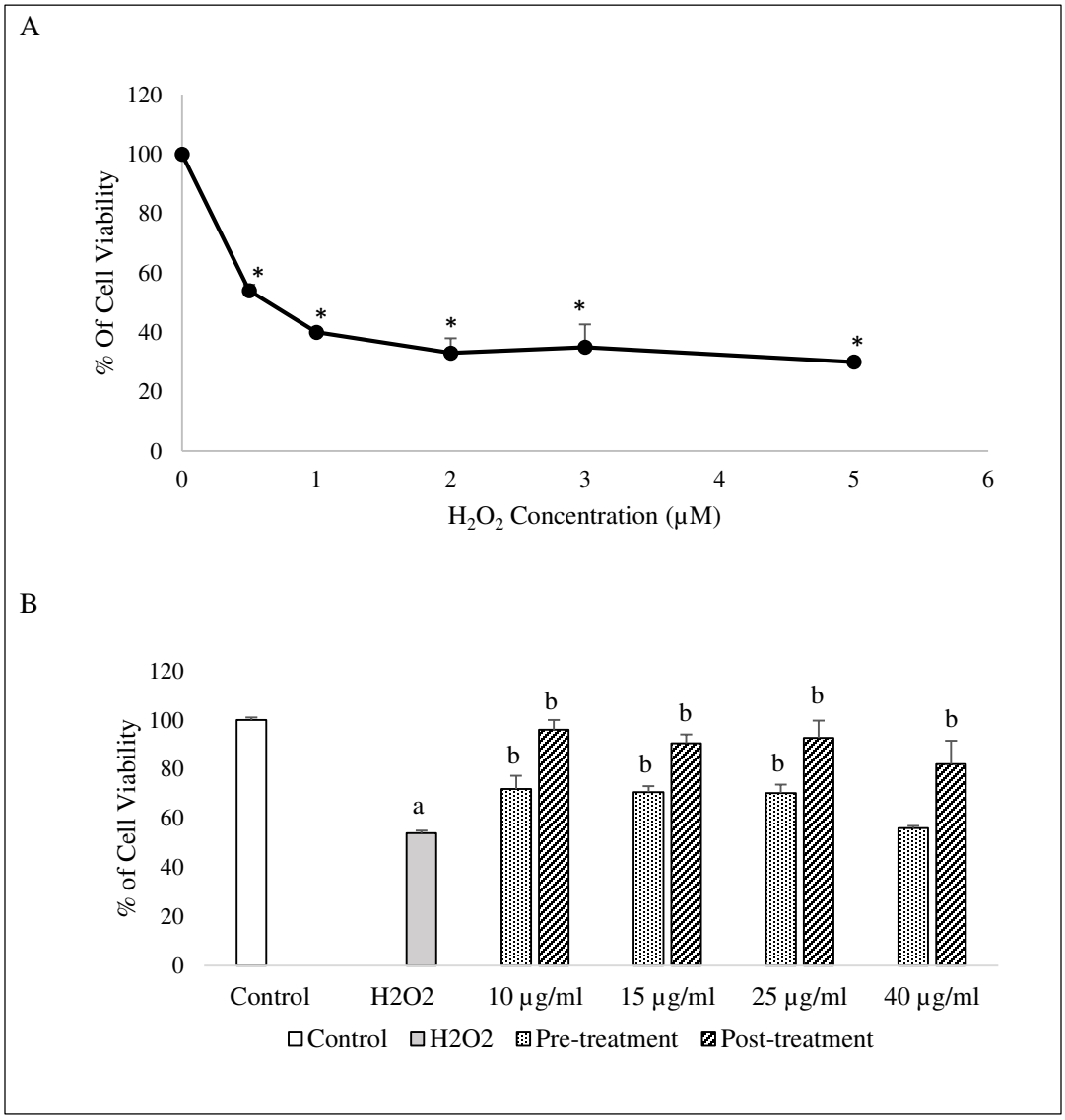

Figure 1 Cell viability in NRCM. A) Effect of different concentration of H_2O_2 (0.5-5 mM) on the cell viability. **B)** Effect of TRF and H_2O_2 IC_{50} on the cell viability. Data are expressed as mean ± SD from three independent experiments (N=3). * indicates significant difference $p < 0.05$ compared to control. [a] indicates significant difference $p<0.05$ compared to control group and [b] indicates significant difference $p<0.05$ compared to H_2O_2 group.

Figure 2(on next page)

Intracellular ROS production

Effect of TRF on H_2O_2- induced ROS production in NRCM. Treatment with TRF significantly reduce H_20_2- indced ROS production Data are expressed as mean ± SD, n = 3, [a] indicates significant difference $p < 0.05$ compared to control . [b] indicates significant difference $p < 0.05$ compared to H_2O_2 group .

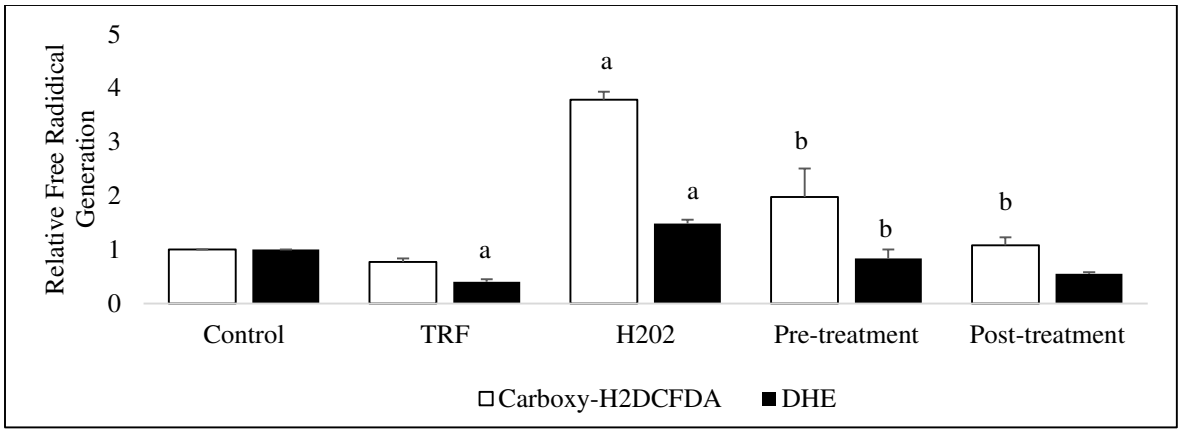

Figure 2 Intracellular ROS production. Effect of TRF on H_2O_2-induced ROS production in NRCM. Treatment with TRF significantly reduce H_20_2-indced ROS production Data are expressed as mean ± SD, n = 3, [a] indicates significant difference $p < 0.05$ compared to control. [b] indicates significant difference $p < 0.05$ compared to H_2O_2 group.

Figure 3(on next page)

LDH activity in the supernatant of NRCM

Effect of TRF on LDH activity of NRCM induced with H_2O_2. Pre-treatment with TRF protect cell from H_2O_2- induced cell injury. Data are expressed as mean ± SD, n = 3, [a] indicates significant difference $p < 0.05$ compared to control . [b] indicates significant difference $p < 0.05$ compared to H_2O_2 group .

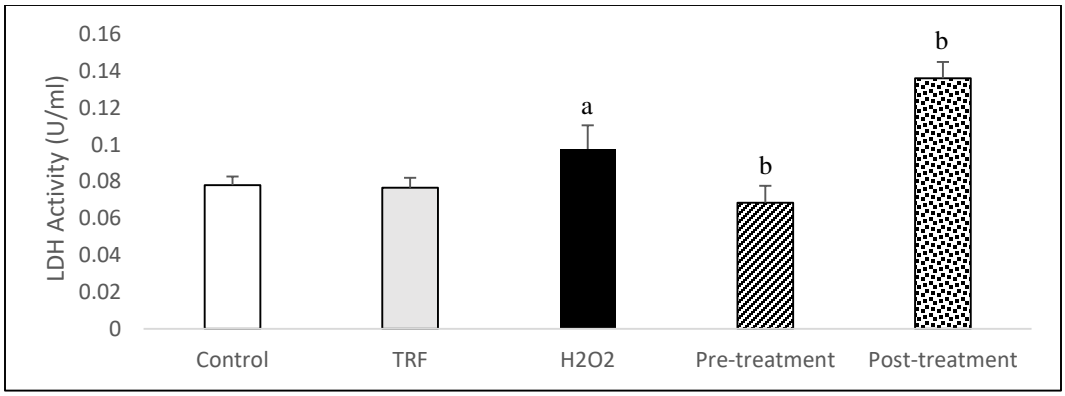

Figure 3 LDH activity in the supernatant of NRCM. Effect of TRF on LDH activity of NRCM induced with H_2O_2. Pre-treatment with TRF protect cell from H_2O_2-induced cell injury. Data are expressed as mean ± SD, n = 3, [a] indicates significant difference $p < 0.05$ compared to control. [b] indicates significant difference $p < 0.05$ compared to H_2O_2 group.

Figure 4(on next page)

Mitochondrial membrane potential changes

A) The effect of TRF on mitochondria membrane potential using microscopic observation by Jc-1 staining. The intensity of J-aggregate (red fluorescence) is higher than J-monomer (green fluorescence) in control and TRF treated group. H_2O_2 exposure increased the intensity of green fluorescence that indicates mitochondrial depolarization. The red fluorescence intensity is increased in pre-treatment with TRF compared to H_2O_2 group showed the protective effect of TRF. Post-treatment showed higher intensity of green fluorescence than red fluorescence indicating mitochondrial is undergoing membrane injury. **B)** Ratio JC-1 aggregate to JC-1 monomer of NRCM. Pre-treatment TRF restored the H_2O_2- mediated decrease in mitochondrial membrane potential. Data are expressed as mean ± SD, n = 4 with [a] indicates significant difference $p < 0.05$ compared to control and [b] indicates significant different $p < 0.05$ compared to H_2O_2 group .

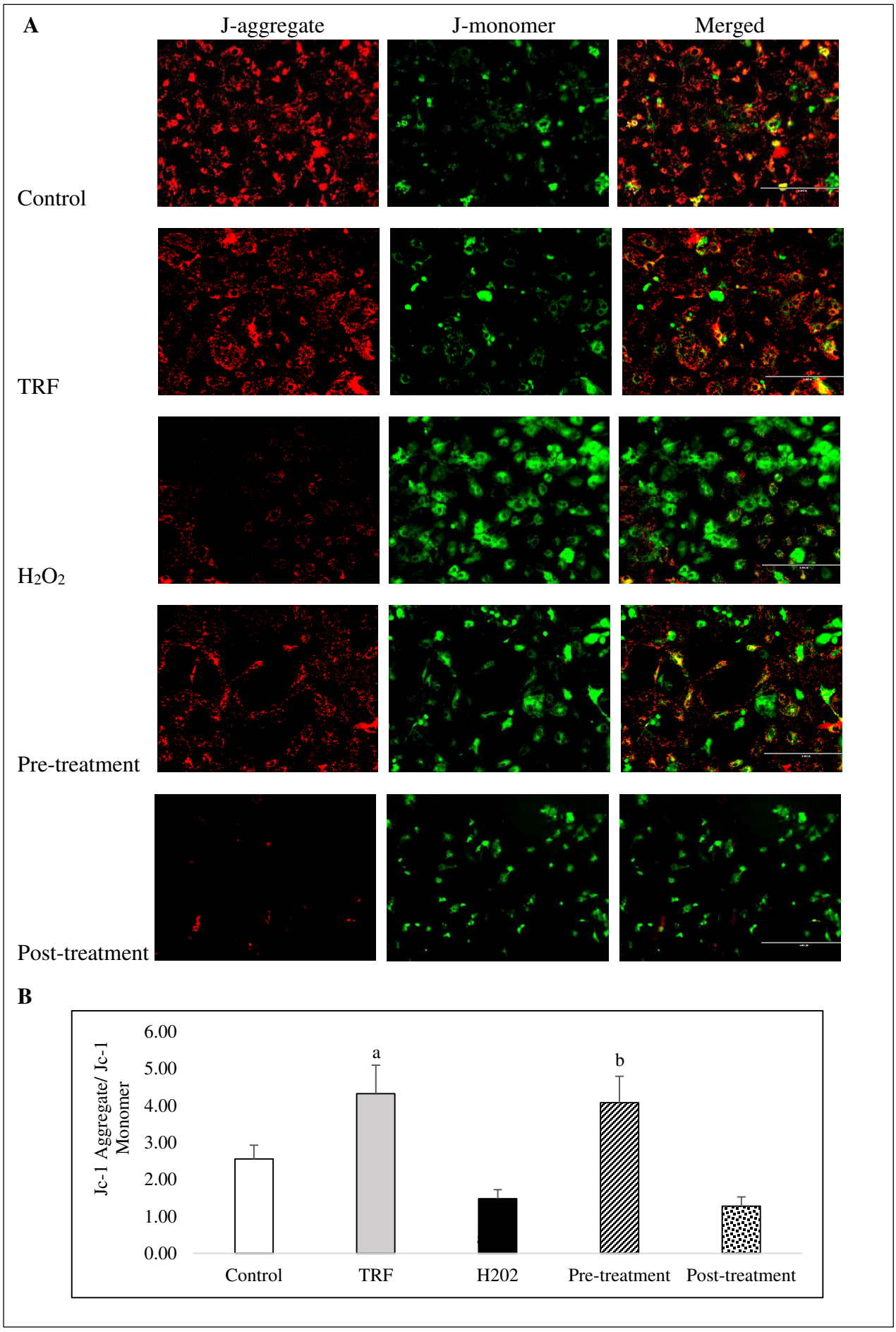

Figure 4 Mitochondrial membrane potential changes. **A)** The effect of TRF on mitochondria membrane potential using microscopic observation by Jc-1 staining. The intensity of J-aggregate (red fluorescence) is higher than J-monomer (green fluorescence) in control and TRF treated group. H_2O_2 exposure increased the intensity of green fluorescence that indicates mitochondrial depolarization. The red fluorescence intensity is increased in pre-treatment with TRF compared to H_2O_2 group showed the protective effect of TRF. Post-treatment showed higher intensity of green fluorescence than red fluorescence indicating mitochondrial is undergoing membrane injury.
B) Ratio JC-1 aggregate to JC-1 monomer of NRCM. Pre-treatment TRF restored the H_2O_2-mediated decrease in mitochondrial membrane potential. Data are expressed as mean ± SD, n = 4 with [a] indicates significant difference $p < 0.05$ compared to control and [b] indicates significant different $p < 0.05$ compared to H_2O_2 group.

Figure 5(on next page)

Percentage of apoptotic cells

The effect of TRF on the apoptosis rate of NRCM induced wit h H_2O_2. Pre-treatment with TRF reduced cell death induced by H_2O_2. Data are expressed as mean ± SD, n = 6 with [a] indicates significance different $p < 0.05$ compared to control group and [b] indicates significance different $p < 0.05$ compared to H_2O_2 group .

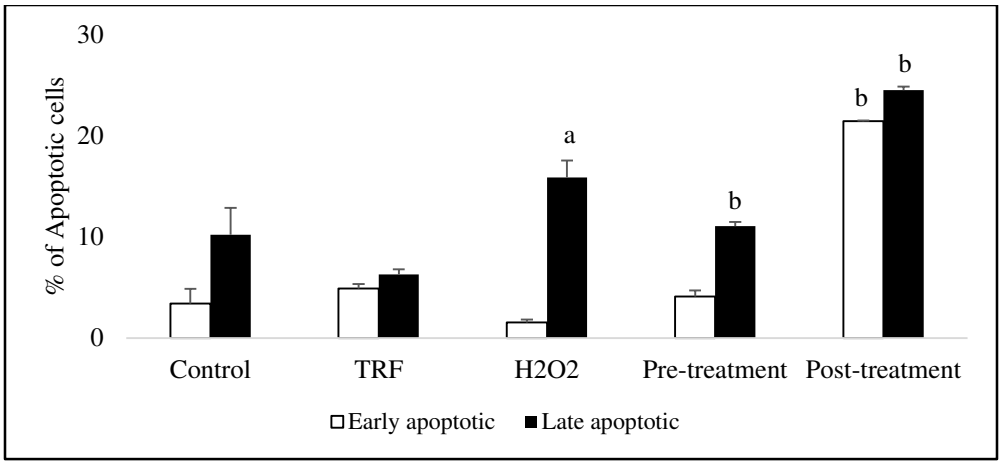

Figure 5 Percentage of apoptotic cells. The effect of TRF on the apoptosis rate of NRCM induced with H_2O_2. Pre-treatment with TRF reduced cell death induced by H_2O_2. Data are expressed as mean ± SD, n = 6 with [a] indicates significance different $p < 0.05$ compared to control group and [b] indicates significance different $p < 0.05$ compared to H_2O_2 group.

Figure 6(on next page)

The effect of TRF on gene expression of antioxidant enzymes

A) GPx B) CAT C) Cu/Zn SOD D) Mn-SOD. Data are expressed as mean ± SD, n = 3. [a] indicates significant different $p < 0.05$ compared to control . [b] indicates significant different $p < 0.05$ compared to H_2O_2 group .

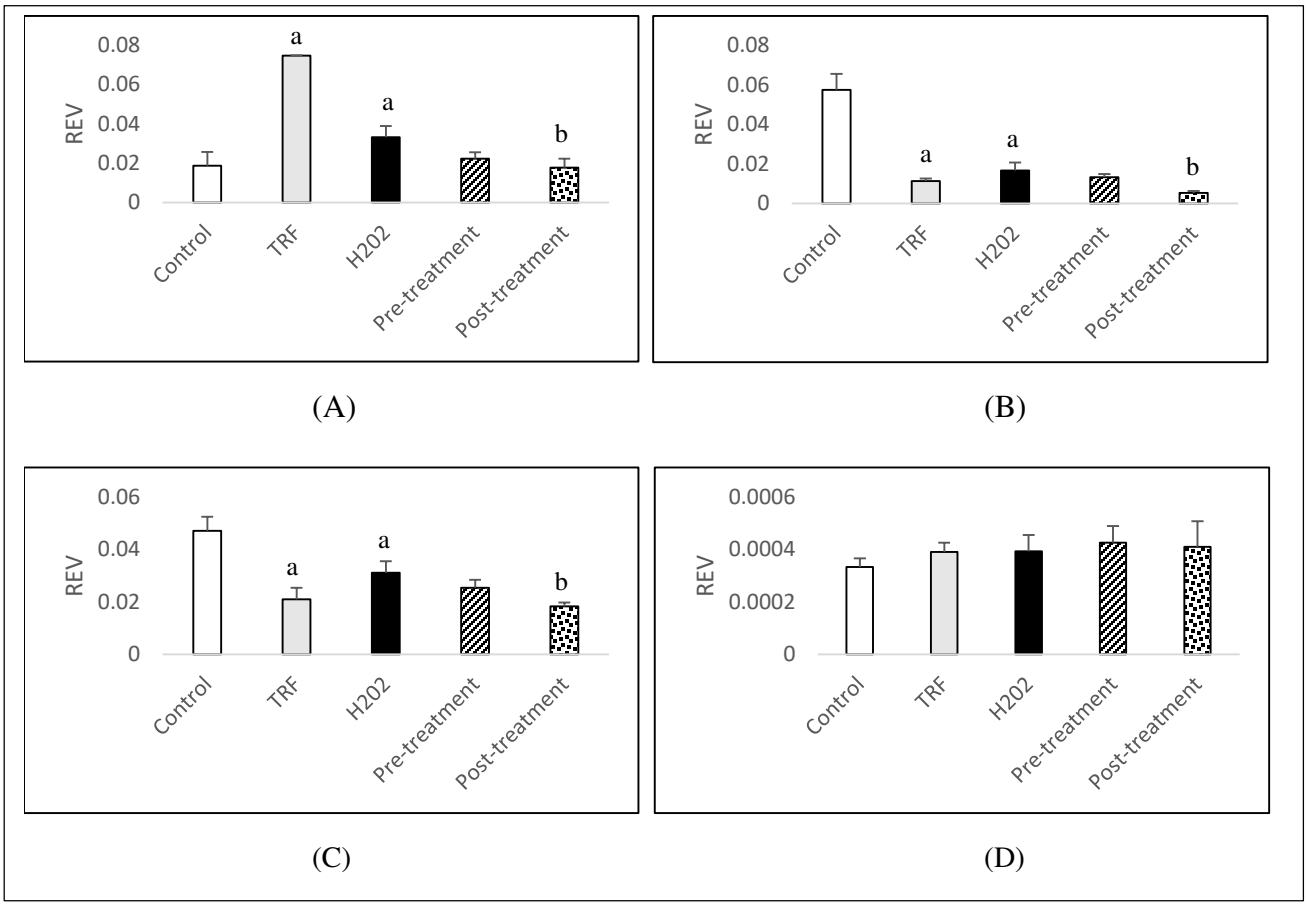

Figure 6 The effect of TRF on gene expression of antioxidant enzymes A) GPx **B)** CAT **C)** Cu/Zn SOD **D)** Mn-SOD. Data are expressed as mean ± SD, n = 3. [a] indicates significant different $p < 0.05$ compared to control. [b] indicates significant different $p < 0.05$ compared to H_2O_2 group.

Figure 7 (on next page)

The effect of TRF on gene expression of apoptosis gene

A) Bax B) Caspase-3 C) Bcl-2. Data are expressed as mean ± SD, n = 3. a indicates significant different $p < 0.05$ compared to control. b indicates significant different $p < 0.05$ compared to H2O2 group.

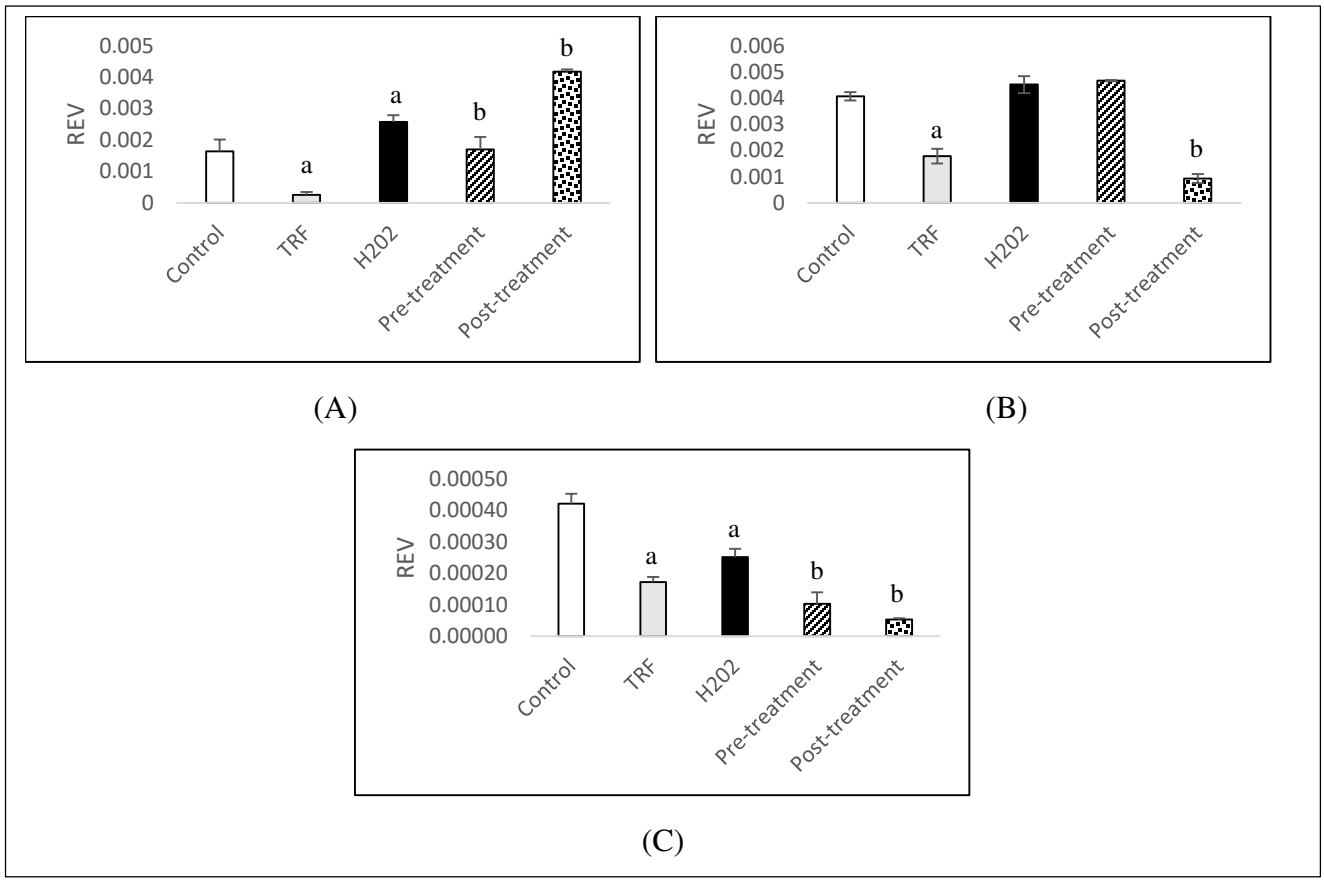

Figure 7 The effect of TRF on gene expression of apoptosis gene. A) Bax B) Caspase-3 C) Bcl-2. Data are expressed as mean ± SD, n = 3. [a] indicates significant different $p < 0.05$ compared to control. [b] indicates significant different $p < 0.05$ compared to H_2O_2 group.

Table 1(on next page)

List of primer sequence

1 Table 1 List of primer sequence

Gene	Forward	Reverse
GAPDH	GTGACTTCAACAGCAACTCC	TGCTCTCAGTATCCTTGCTG
GPx1	CCTCAAGTATGTCCGACCCG	GATGTCGATGGTGCGAAAGC
CAT	GGTAACTGGGACCTTGTGGG	CATCTGGAATCCCTCGGTCG
MnSOD	CCTCAGCAATGTTGTGTCGG	TCGTGGTACTTCTCCTCGGT
Cu/Zn SOD	TCCTAGACTGACGCTTCCCA	CTGTGGAGTGCATAGGTGTGA
Caspase-3	GAGCTTGGAACGCGAAGAAAA	CCATTGCGAGCTGACATTCC
Bax	TGGCGATGAACTGGACAACA	TAGGAAAGGAGGCCATCCCA
Bcl-2	CATCTCATGCCAAGGGGGAA	CAGTATCCCACTCGTAGCCC

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3